

1 DIAGNOSTIC METHODS FOR CONGESTIVE HEART FAILURE

2
3 FIELD OF THE INVENTION

4 The instant invention relates generally to the field of
5 immunology; particularly to the use of immunologic assays to
6 diagnose abnormal or disease states and most particularly to
7 a sandwich ELISA (enzyme-linked immunosorbent assay) assay
8 for the quantification of a truncated glycoporphin circulating
9 in biological fluid which is diagnostic for congestive heart
10 failure (CHF).

11
12 BACKGROUND OF THE INVENTION

13 The diagnosis of a given disease requires standard
14 agreed-upon observations usually made by the attending
15 physician of the sick patient. For some diseases, a single
16 test is available which gives nearly definitive results
17 sufficient for a correct diagnosis, for example, the glucose
18 tolerance test for diabetes. However, most diseases require a
19 number of sophisticated tests to arrive at a probable
20 diagnosis. At the present time, therapeutic interventions are
21 frequently initiated at late stages of disease, often
22 resulting in only modest improvements in the quality and
23 length of the affected patients life. Disease prevention is
24 easier and more effective than disease therapy. Earlier

1 diagnosis decreases disease-associated morbidities,
2 increases the quality and length of life of the patient and
3 decreases overall costs of health care. Thus, it is a goal of
4 biomedical researchers to develop diagnostic tests which can
5 correctly diagnose disease at the early stages.

6 Early diagnosis of congestive heart failure (CHF) is
7 particularly beneficial since the cardiac re-structuring
8 which occurs with progressive disease may be slowed or
9 prevented with early therapeutic intervention. However, early
10 diagnosis has proven elusive since symptoms generally do not
11 appear until the heart has already suffered structural
12 changes.

13 CHF is a serious condition with a high mortality rate
14 affecting approximately five million Americans (see US
15 6,572,895 for a discussion of CHF). It is currently believed
16 that CHF is not a distinct disease process in itself, but
17 rather represents the effect of multiple abnormalities which
18 interact together to ultimately produce the progressive loss
19 of the ability of the heart to function as a circulatory
20 pump. Major pathophysiologic abnormalities which occur in CHF
21 are activation of the hypothalamic-pituitary-adrenal axis,
22 systemic endothelial dysfunction and myocardial re-
23 structuring. The progression of CHF can be initiated by an
24 event such as myocardial infarction wherein the heart muscle

1 is damaged or it can result from hypertension and/or cardiac
2 malformations. Recently, it has been discovered that patients
3 with certain conditions such as insulin resistance and Type
4 II diabetes have a particularly high risk for heart failure
5 and poor prognosis once they develop CHF (Soläng et al.
6 European Heart Journal 20:789-795 1999).

7 Disease processes, such as those which occur in diabetes
8 and CHF, often result in cellular and/or tissue damage
9 followed by the release of cellular and/or tissue specific
10 biopolymer markers into the bodily fluids of an individual.
11 These biopolymer markers are harbingers of disease and/or
12 disease progression. Association of such biopolymer markers
13 with abnormal and/or disease states provides new diagnostic
14 avenues which may allow identification of patients in the
15 early stages of disease or patients at risk for developing
16 disease. Identification of biopolymer markers diagnostic for
17 CHF is particularly advantageous considering the progressive
18 pathophysiology involved in CHF. What is lacking in the art
19 is an efficient, easy to perform diagnostic method capable of
20 identifying an individual suffering from CHF.

21 22 SUMMARY OF THE INVENTION

23 The instant invention provides an efficient, easy to
24 perform diagnostic method capable of identifying an

1 individual suffering from CHF. The method comprises a
2 sandwich ELISA assay using mouse monoclonal antibodies(anti-
3 glycophorins)to quantify elevated glycophorin in biological
4 fluids. Glycophorin is the major integral membrane protein of
5 the mammalian red blood cell (RBC) and is highly
6 glycosylated. The glycosylation of glycophorin is responsible
7 for the overall negative charge of the RBC cellular surface
8 leading to the normal electrostatic repulsion among red blood
9 cells. In the disease processes of diabetes and CHF the red
10 blood cell (RBC) membrane proteins, including glycophorins,
11 are abnormally degraded, thus reducing the overall negative
12 charge of the cellular surface leading to a decrease in the
13 normal electrostatic repulsion among red blood cells. As a
14 consequence, aggregation of red blood cells occurs in the
15 pathogenesis of diabetes and CHF. Using the sandwich ELISA
16 assay of the invention, the instant inventors identified an
17 abnormal, circulating glycophorin in the plasma of CHF
18 patients. This glycophorin had a lower molecular weight than
19 that of normal glycophorin, thus it is predicted to be a
20 truncated fragment which has been cleaved from the RBC
21 membrane surface during the disease process.

22 Three mouse monoclonal antibodies are used in the ELISA
23 assay of the instant invention; 3F4, 6G4 and 5F4. Monoclonal
24 antibody 3F4 recognizes amino acid residues 5-25 of SEQ ID

1 NO:2 and SEQ ID NO:4 (glycophorins A and B). Monoclonal
2 antibody 6G4 recognizes amino acid residues 39-45 of SEQ ID
3 NO:2 (glycophorin A). Monoclonal antibody 5F4 recognizes
4 amino acid residues 107-119 of SEQ ID NO:2 (glycophorin A).

5 Accordingly, it is an objective of the instant invention
6 to provide a sandwich ELISA assay using mouse anti-
7 glycophorin monoclonal antibodies 3F4, 6G4 and 5F4 for the
8 quantification of an abnormal, truncated glycophorin
9 circulating in biological fluid.

10 It is another objective of the instant invention to
11 identify a circulating, truncated glycophorin diagnostic for
12 congestive heart failure (CHF).

13 Other objectives and advantages of this invention will
14 become apparent from the following description taken in
15 conjunction with the accompanying drawings wherein are set
16 forth, by way of illustration and example, certain
17 embodiments of this invention. The drawings constitute a
18 part of this specification and include exemplary embodiments
19 of the present invention and illustrate various objects and
20 features thereof.

21
22 BRIEF DESCRIPTION OF THE FIGURES

23 FIGURE 1 shows the data resulting from the sandwich
24 ELISA using monoclonal antibody 3F4.

FIGURE 2 shows the data resulting from the sandwich ELISA using monoclonal antibodies 6G4, 5F4 and 3F4.

FIGURE 3 shows the data resulting from the direct ELISA evaluating the presence of an autoantibody to glycophorin.

FIGURE 4 shows the results of immunoprecipitation of glycophorin from the plasma of CHF patients.

FIGURES 5A-C show chromatograms; FIGURE 5A shows captured glycophorin from CHF patients; FIGURE 5B shows captured glycophorin from healthy patients and FIGURE 5C shows captured purified glycophorin.

FIGURE 6 shows chromatograms after deglycosylation treatment; the top chromatograph shows purified glycophorin; the middle chromatograph shows captured glycophorin from CHF patients and the bottom chromatograph is a control run without a glycophorin sample.

DEFINITIONS

The following list defines terms, phrases and abbreviations used throughout the instant specification. Although the terms, phrases and abbreviations are listed in the singular tense the definitions are intended to encompass all grammatical forms.

As used herein, the abbreviation "CHF" refers to congestive heart failure.

1 As used herein, the abbreviation "GP" refers to
2 glycophorin.

3 As used herein, the abbreviation "GPA" refers to
4 glycophorin A.

5 As used herein, the abbreviation "GPB" refers to
6 glycophorin B.

7 As used herein, the abbreviation "GPAX2" refers to the
8 dimerized form of glycophorin A.

9 As used herein, the abbreviation "GPBX2" refers to the
10 dimerized form of glycophorin B.

11 As used herein, the abbreviation "ELISA" refers to
12 enzyme-linked immunosorbent assay.

13 As used herein, the abbreviation "RBC" refers to red
14 blood cell.

15 As used herein, the abbreviation "MoAb" refers to
16 monoclonal antibody.

17 As used herein, the abbreviation "MS" refers to mass
18 spectrometry.

19 As used herein, the abbreviation "SELDI" refers to a
20 mass spectrometric technique; surface enhanced laser
21 desorption ionization.

22 As used herein, the abbreviation "PBS" refers to
23 phosphate buffered saline.

24 The terms "RBC", "red blood cell" and "erythrocyte" are

1 used interchangeably herein.

2 As used herein, the term "glycophorin" refers to the
3 major integral glycoprotein of the mammalian erythrocyte
4 membrane. Glycophorin is highly glycosylated and occurs in
5 isoforms A and B (see Concise Encyclopedia: Biochemistry and
6 Molecular Biology, Third Edition, Revised and Expanded by
7 Thomas A. Scott and E. Ian Mercer, Walter de Gruyter, Berlin-
8 New York 1997, pages 201-202 and Instant Notes: BioChemistry,
9 2nd edition, B.D. Hames and N.M. Hooper, Springer-Verlag New
10 York 2000, pages 125, 126 and 130 for an introduction to the
11 RBC membrane and glycophorins).

12 As used herein, the term "circulating, truncated
13 glycophorin" refers to the abnormal glycophorin fragment
14 identified by the assay of the instant invention in the serum
15 of CHF patients. The 3F4 mouse anti-glycophorin monoclonal
16 antibody which recognizes the extracellular portion of
17 glycophorin A and B binds to this circulating, truncated
18 glycophorin. This circulating, truncated glycophorin is
19 structurally different from the normal soluble glycophorin
20 and is theorized to be a fragment cleaved from the RBC
21 surface during disease processes.

22 As used herein, the term "biological fluid" refers to
23 any bodily fluid. Illustrative, albeit non-limiting examples
24 are blood, blood products, urine, saliva, cerebrospinal fluid

1 and lymphatic fluid.

2 As used herein, the term "subject" refers to any
3 mammalian organism. A particularly preferred subject is a
4 human.

5 As used herein, the term "corresponding" is used
6 generally with reference to antibody-antigen binding
7 complexes, for example, an antibody corresponding to an
8 antigen will bind to the antigen under physiologic
9 conditions. The bound antibody-antigen is referred to as an
10 antibody-antigen binding complex.

11 As used herein, the term "signal generating substance"
12 refers to any material which undergoes a measurable reaction.
13 Illustrative, albeit non-limiting examples are fluorophores,
14 enzymes and radioisotopes. A particularly preferred signal
15 generating substance is peroxidase, the use of which is
16 illustrated in the examples herein.

17 As used herein, the term "congestive heart failure"
18 refers to a progressive, debilitating condition wherein the
19 heart loses its ability to function as a circulatory pump.

20 As used herein, the term "antibody" refers to a protein
21 secreted by B lymphocytes capable of binding specific
22 molecules under physiologic conditions.

23 As used herein, the term "monoclonal antibody" refers to
24 an antibody having single epitope specificity.

1 As used herein, the term "polyclonal antibody" refers to
2 an antibody capable of binding with multiple epitopes.

3 As used herein, the term "antigen" broadly refers to any
4 substance which induces an immune reaction; more particularly
5 the term "antigen" refers to the corresponding binding
6 partner of an antibody.

7 As used herein, the term "auto-antibody" refers to an
8 antibody which recognizes self antigens, for example,
9 antibodies produced by an organism which bind the organism's
10 own proteins are referred to as auto-antibodies.

11 Specific antibodies can be used to quantify the amount
12 of corresponding antigen in a biological sample. As used
13 herein, the term "ELISA" refers to an enzyme-linked
14 immunosorbent assay which can quickly detect and quantify
15 minute amounts (less than a nanogram) of antigen in a
16 biological sample. The test antibody is bound to an inert
17 polymer support, such as a plastic tray with molded wells,
18 and then exposed to the biological sample. Unbound proteins
19 are washed away and a second antibody that reacts with the
20 antigen at a different epitope than the test antibody reacts
21 with is added. This second antibody has an enzyme attached to
22 it that converts a colorless or nonfluorescent substrate into
23 a colored or fluorescent product. The amount of second
24 antibody bound, and hence the amount of protein antigen

present in the original biological sample, is determined by the quantification of the intensity of color or fluorescence produced. This ELISA assay is also referred to as an "indirect ELISA" or a "sandwich ELISA". (see Instant Notes: BioChemistry, 2nd edition, B.D. Hames and N.M. Hooper, Springer-Verlag New York 2000, pages 112-114 for an introduction to the general principles of ELISA assays). There is also a form of ELISA assay that is referred to as "direct" wherein the antigen is bound to an inert polymer support and exposed to a biological sample containing the corresponding antibody.

DETAILED DESCRIPTION OF THE INVENTION

As a result of disease processes, damage to cells and tissues of the body occurs at the cellular and sub-cellular levels. Initially, these processes may only cause damage to the outer membranes of cells, causing a sloughing off of portions of the exterior cellular matrices, which process is broadly defined as reversible damage. As the length of time and/or the severity of the disease condition increases, the outer membranes begin to break down, resulting in membrane rupture followed by the release of intra-cellular components, which process is broadly defined as irreversible damage. When such damage occurs (reversible or irreversible), biopolymer

1 markers are released into the circulation, causing the immune
2 system to become activated, since these biopolymer markers
3 are not normally present in the bodily fluids. The immune
4 system views these biopolymer markers as invading pathogens
5 or foreign bodies whose threat must be neutralized. In an
6 effort to persevere against this perceived threat, auto-
7 antibodies are formed to these biopolymer markers. These
8 auto-antibodies can be characterized as sequela which are
9 indicative of the original damaging insult to the organism.
10 The presence of the auto-antibodies validates the theory that
11 cellular damage acts as an initiator of an immune response
12 leading to a cascade of auto-antibody production which
13 ultimately manifests itself in a characteristic and often
14 predictable disease state. The appearance of these biopolymer
15 markers and their associated auto-antibodies are harbingers
16 of disease and/or disease progression and are useful for
17 diagnostic purposes.

18 Damage to the red blood cell membrane is known to occur
19 in disease processes such as diabetes and CHF. In these
20 diseases there is an increase in enzyme production and/or
21 activation (neutrophil proteases, metalloproteases,
22 sialidases and endopeptidases) that directly and/or indirectly
23 leads to abnormal degradation of red blood cell membrane
24 proteins (Gaczyńska et al. Cytobios 75:7-11 1993; Venerando

1 *et al.* Blood 99(3):1064-1070 2002; Wegner *et al.*
2 Cardiovascular Research 31:891-898 1996; Piwowar *et al.*
3 Clinical Chemistry Lab Medicine 38(12):1257-1261 2000 and
4 Santos-Silva *et al.* Clinica Chimica Acta 320:29-35 2002).

5 Additionally, it is well-documented that erythrocyte
6 (RBC) aggregability is increased in diabetes and in vascular
7 atherosclerotic disease (Caimi *et al.* Thromb Haemost 83:516-
8 517 2000; Demiroglu *et al.* Experimental Clinical Endocrinol
9 Diabetes 107(1):35-39 1999; Martínez *et al.* Clinical
10 Hemorheology and Microcirculation 18:253-258 1998 and Ziegler
11 *et al.* Metabolism 43(9):1182-1186 1994). Alterations in RBC
12 membrane phospholipids are associated with RBC aggregability
13 (Martínez *et al.* Clinical Hemorheology and Microcirculation
14 18:253-258 1998). Sphingomyelin is the main phospholipid of
15 the outer membrane and has been shown to contain a greater
16 percentage of saturated fatty acids in diabetic patients than
17 in non-diabetic patients. This increase in saturation is
18 thought to reduce electrostatic repulsion between red blood
19 cells, which in turn increases their aggregability.

20 Loss of glycophorins further reduces the electrostatic
21 repulsion of red blood cells. Glycophorin is the major RBC
22 integral membrane glycoprotein. The high sialylation of
23 glycophorin is responsible for the negative surface charge
24 which leads to the normal electrostatic repulsion between red

1 blood cells (Eylar et al. The Journal of Biological Chemistry
2 237(6):1992-2000 1962). The increase in enzyme production
3 and/or enzyme activation in disease processes such as
4 diabetes results in the loss of glycoporphins from the RBC
5 membrane. These glycoporphin fragments are released into the
6 bodily fluids where they stimulate the production of auto-
7 antibodies. The decrease in glycoporphin in turn leads to a
8 decrease in the normal negative charge of the RBC membrane
9 surface and thus decreases the overall electrostatic
10 repulsion between blood cells. Loss of the electrostatic
11 repulsion between red blood cells results with the
12 aggregation of red blood cells seen in diabetes.

13 Without being bound by any particular theory, the
14 instant inventors propose that the circulating, truncated
15 glycoporphin identified in the plasma of CHF patients using
16 the sandwich ELISA assay described herein is an extracellular
17 glycoporphin fragment which has been cleaved from the RBC
18 membrane during the disease process. This circulating,
19 truncated glycoporphin is structurally different from the
20 normal soluble form of glycoporphin. The mouse anti-
21 glycoporphin 3F4 monoclonal antibody which recognizes amino
22 acid residues 5-25 of SEQ ID NO:2 and SEQ ID NO:4
23 (glycoporphins A and B) also recognizes the circulating,
24 truncated glycoporphin. The instant inventors have also shown

1 by direct ELISA assay that CHF patients show an increase in
2 anti-glycophorin auto-antibodies. Thus, it is concluded that
3 this circulating, truncated glycophorin can be used as a new
4 biopolymer marker for CHF diagnosis.

5 6 EXPERIMENTAL PROCEDURES

7 SEQUENCES

8 Homo sapiens (human) glycophorin A nucleic acid sequence
9 is disclosed as SEQ ID NO:1 and translates into glycophorin A
10 protein disclosed as amino acid sequence SEQ ID NO:2. Homo
11 sapiens (human) glycophorin B nucleic acid sequence is
12 disclosed as SEQ ID NO:3 and translates into glycophorin B
13 protein disclosed as amino acid sequence SEQ ID NO:4.

14 15 ANTIBODIES

16 The mouse anti-glycophorin monoclonal antibodies used in
17 the following experiments were purchased from BioAtlantic.
18 Monoclonal antibody 6G4 recognizes amino acid residues 39-45
19 of SEQ ID NO:2 (glycophorin A). Monoclonal antibody 5F4
20 recognizes the intracellular portion of glycophorin A
21 comprising amino acid residues 107-119 of SEQ ID NO:2.
22 Monoclonal antibody 3F4 recognizes the extracellular portion
23 of glycophorins A and B amino acid residues 5-25 of SEQ ID
24 NO:2 and SEQ ID NO:4. The binding of the 3F4 antibody to its

1 epitope is sugar-dependent whereas the binding of the 6G4
2 antibody is not. These monoclonal antibodies are described in
3 detail in Rasamoeliso et al. Vox Sanguinis 72:185-191 1997.

4 The mouse anti-glycophorin 3F4 monoclonal antibody was
5 deposited with the American Type Culture Collection (ATCC) on
6 April 23, 2000 as hybridoma NaM26-3F4D11A2 under Accession
7 number PTA-5154. The American Type Culture Collection (ATCC)
8 is located at 10801 University Boulevard, Manassas, Virginia
9 20110-2209.

10 11 QUANTIFICATION OF GLYCOPHORIN BY SANDWICH ELISA

12 One ug of each MoAb in 100ul of 50mM carbonate pH 9.4
13 was coated on ELISA plates (Nuc, Denmark) and set overnight
14 at +4°C. Plates were then washed 3 times with 0.01M phosphate
15 buffer 150mM NaCl pH 7.4 (PBS) purchased from Sigma
16 containing 0.05% Tween 20 (PBST). Plates were then blocked
17 with 200ul of PBST containing 0.5% BSA (Sigma) for 30 minutes
18 at 37°C. 100ul of CHF patient plasma (PRAISE 2 study) and
19 healthy control plasma (Intergen) diluted 1/10 in PBST were
20 then added per well in duplicate and incubated for 2 hours at
21 room temperature. After 3 washes with PBST, 100ul of rabbit
22 polyclonal anti-glycophorin A+B (BioAtlantic) were added and
23 incubated for 1 hour at room temperature followed by the
24 addition of 100ul of peroxidase labeled donkey polyclonal

1 anti-rabbit IgG (H+L) diluted 1/50,000 in PBST containing
2 0.5% BSA (Jackson ImmunoResearch). The presence of the
3 captured glycoporphins is detected by adding 100ul of TMB
4 (Moss, Inc.). The reaction was stopped with 50ul of 1N H₂SO₄.
5 Plates were then read at 450nm on the BioRad microplate
6 reader.

7 Figure 1 shows the result of the sandwich ELISA using the
8 3F4 monoclonal antibody. The absorbance at 450 nm is shown on
9 the Y axis. Glycophorin captured from the plasma of CHF
10 patients is shown on the left and the glycophorin captured from
11 normal plasma (control, n=36) is shown on the right. The signal
12 is significantly higher in CHF plasma than in controls
13 (p<0.001) calculated by an independent t- test indicating a
14 higher amount of glycoporphins in CHF plasma samples. The 3F4
15 MoAb recognizes the common sequence on both glycoporphins A and
16 B (amino acid residues 5-25 of SEQ ID NO:2 and SEQ ID NO:4).
17 This binding is sugar-dependent since this fragment of
18 glycophorin is highly glycosylated.

19 In order to ascertain whether the assay is specific to
20 the extracellular polypeptide of glycophorin or the
21 oligosaccharide chains, the MoAbs 6G4 (recognizes amino acid
22 residues 39-45 of SEQ ID NO:2) and 5F4 (recognizes amino acid
23 residues 112-129 of SEQ ID NO:2) were used. Both bind to the
24 glycophorin A backbone independently of the sugar chains.

Eight CHF samples having the most elevated amount of glycophorin and 8 normal plasma samples having the lowest amount of glycophorin were analyzed and the result is shown in Figure 2. Figure 2 shows results from sandwich ELISA assays comparing the glycoprotein captured in plasma from CHF patients and the glycoprotein captured in normal control plasma (n=8). The top panel shows results using the 6G4 MoAb (p=0.001); the middle panel shows results using the 5F4 MoAb (p=0.36) and the bottom panel shows the results using the 3F4 MoAb (p=0.003). The Y axis represents the absorbance read at 450nm. Glycophorin captured from the plasma of CHF patients is shown on the left and the glycoprotein captured from normal plasma is shown on the right in all three panels. The result shows that 6G4 detects elevated amount of glycoprotein in CHF samples, while 5F4 shows no significant difference between both CHF and normal human plasma. This result indicates that glycoprotein may be cleaved from the red blood cell membrane during the progression of CHF since the fragments recognized by the antibodies are extracellular fragments. However, it is noted that a soluble form of glycoprotein is present in normal as well as CHF patient plasma that is detected by the 5F4 monoclonal anti-intracellular domain of glycoprotein.

DETECTION OF AUTO-ANTIBODY BY DIRECT ELISA

0.5ug of purified glycophorin from blood group MM or asialoglycophorins from blood group MN (Sigma) in 50mM carbonate buffer pH 9.4 was adsorbed onto ELISA plates overnight at +4°C. Plates washed 3 times with 0.01M Phosphate buffer 150mM NaCl pH 7.4 (PBS) from Sigma containing 0.05% Tween 20 (PBST). Plates were then blocked with 200ul of PBST containing 0.5% BSA (Sigma) for 30 minutes at 37°C. 100ul of CHF plasma (PRAISE 2 study) and normal control plasma (Intergen) diluted 1/100 in PBST were then added per well in duplicate and incubated for 2 hours at room temperature. After 3 washes with PBST, 100ul of peroxidase labeled goat polyclonal anti-human IgG (H+L) diluted 1/10,000 in PBST (Jackson ImmunoResearch) were added. The presence of auto-antibody anti-glycophorins was detected by adding 100ul of TMB (Moss, Inc.) and the reaction was stopped with 50ul of 1N H₂SO₄. Plates were read at 450 nm on the BioRad microplate reader.

Glycophorin is known to be highly immunogenic due to the presence of a high amount of sugar chains. Once found in plasma it may induce an immune response generating anti-glycophorin auto-antibody.

To demonstrate the presence of CHF-induced auto-antibody against glycoporphin, glycoporphins from blood group MM and asialo glycoporphins from blood group MN were coated on ELISA

plates and plasma from healthy donors or from CHF patients were added. Figure 3 shows the results of the direct ELISA assay evaluating the presence of a CHF-induced auto-antibody in the plasma of normal and CHF patients (n=36). In the top panel, glycophorin from blood group MN was coated on the plate (p=0.01) and the bottom panel, desialylated glycophorin from blood group MN was coated on the plate (p=0.03). The Y axis represents the absorbance read at 450nm. Figure 3 shows the presence of auto-antibodies in CHF; independent to the blood group (M or N) and the heavy sialic acids on glycophorin.

IDENTIFICATION OF GLYCOPHORINS IN CHF PLASMA BY IMMUNOPRECIPITATION AND DETECTION BY IMMUNOBLOTTING

1.2ml of pooled CHF plasma from the PRAISE 2 study was diluted v/v with PBS containing 0.5% Triton X-100. Then 2ul of 3F4 MoAb at 1.7 mg/ml were added. After overnight incubation at +4°C, 25 ul of goat IgG anti-mouse IgG (H+L) coupled to SEPHAROSE-4B beads (Zymed) were added. The mixture was incubated for 5 hours at +4°C and then the beads were washed 3 times with PBS containing 0.05% Tween 20. The captured (glyco)protein was eluted with 100ul of 0.1M glycine pH 2.5 then neutralized with 1M Tris pH 11. The eluate was concentrated on CentriVap Concentrator (Labconco), resuspended

1 in 50ul of SDS-PAGE sample buffer, boiled 5 minutes at 100°C
2 and then loaded on 10% SDS-PAGE gel. At the end of the
3 electrophoresis, proteins were transferred onto a
4 nitrocellulose membrane and stained with 3F4 MoAb anti-GPA+B
5 followed by a peroxidase labeled goat polyclonal anti-mouse IgG
6 (H+L) diluted 1/50,000 in PBST (Jackson ImmunoResearch) . The
7 immunoblot was then developed using ECL (Amersham Pharmacia).
8 To control the cross-reactivity of the secondary antibody to
9 the 3F4 eluted from the column, the blot was incubated with the
10 secondary antibody alone.

11 The molecules captured by 3F4-column were eluted and loaded
12 on 10% SDS-PAGE gel and assessed on immunoblotting against the
13 same MoAb. As shown in figure 4, the glycophorins found in CHF
14 plasma have a molecular weight of 75, 45 and 40 kDa (lane 2,
15 blot incubated with 3F4). Usually glycophorins run at 80 - 70
16 - 40 - 37 and 20kDa as dimer form of GPA, dimer GPA/GPB, dimer
17 form of GPB, monomer form of GPA and monomer form of GPB,
18 respectively as shown on lane 1 loaded with normal glycophorin
19 purified from normal red blood cell membrane. Thus, the
20 glycophorins found in the plasma of CHF patients have different
21 molecular weights as compared to the normal glycophorin
22 purified from RBC membranes. The immunoblot was incubated with
23 the secondary antibody alone (control) or with the 3F4 antibody
24 and then the secondary antibody. Lane 1 (in both blots) shows

glycophorin purified from RBC membranes and Lane 2 (both blots) shows glycophorin from CHF patient plasma. Protein markers from 25 to 200 kDaltons are shown on the far left.

The IgG identified in control and 3F4 blots is the mouse monoclonal 3F4 used for the immunoprecipitation and released from the column. A band with a high MW > 200kDa is also detected. The instant inventors are not sure about the nature of this band. The band may be a complex form of IgM or IgG autoantibodies and the glycophorins.

IDENTIFICATION OF GLYCOPHORIN IN CHF PATIENT SAMPLE BY SELDI-TOF

The method of the instant invention can be carried out using the techniques of mass spectrometry. The PS20 chip (Ciphergen) was washed with pure Acetonitrile-190 (ACN) (Caledon) and allowed to air dry. 50 µg of Protein G (Pierce) was dissolved in 50µl UF water and 1ul was loaded to each spot containing 1µl of ACN. The mixture was incubated 1 hour in a humidity chamber and then the spot was blocked with 10µl of 0.5M Tris-HCl pH 7.4 (Caledon) for 15 minutes. The chip was then washed with UF water and allowed to air dry. Monoclonal antibody (MoAb) anti-GPA+GPB, the 3F4 at 1.7mg/ml (BioAtlantic) was diluted 1/3 in PBS containing 0.1% TRITON X (Sigma) and 3µl of the MoAb solution was loaded per spot and incubated for 1

1 hour in a humidity chamber. Unbound MoAb was washed away from
2 the chip by washing with PBS.

3 Purified glycophorin (Sigma), CHF plasma from PRAISE 2
4 study or normal plasma (Intergen) was added to the 3F4-coated
5 chip as follows:

6 The glycophorin at 1mg/ml was diluted 1/5 in PBS; CHF and
7 normal plasma samples were diluted 1/5 in PBS containing 0.05%
8 Tween 20, and 2µl of each were loaded per spot. The chip was
9 then incubated for 1 hour in a humidity chamber and washed
10 twice with UF water.

11 The captured glycophorin was then treated with Endoproteinase
12 GluC (Roche Diagnostics). For that, the GluC powder was
13 dissolved in 50µl of UF water and a 1/10 dilution in 50mM
14 Ammonium Carbonate pH 7.8 (BDH Laboratory Supplies) was
15 prepared. 1µl of the GluC solution was added to each spot and
16 incubated 2 hours in a humidity chamber. The spot was then
17 allowed to dry and was either treated using Calbiochem
18 deglycosylation kit or directly analyzed on SELDI after adding
19 1ul of saturated sinapinic acid (Sigma) in 0.5% TFA 50% ACN.
20 The chip was then read on SELDI (Ciphergen) at a
21 Sensitivity=10, Intensity=180-190, range of 0-5000 Da
22 (optimized for 0-5000 Da).

23 The (glyco)protein captured on the 3F4 chip was treated by
24 GluC. Figure 5A shows data resulting from the on-chip treatment

1 of the captured glycophorin from CHF. Figure 5B shows data
2 resulting from the on-chip treatment of the normal plasma
3 samples. Figure 5C shows data resulting from the on-chip
4 treatment of purified glycophorin. As shown in Figures 5A-C, a
5 (glyco)peptide with a m/z of 2361+H is found in both CHF and
6 glycophorin demonstrating that the (glyco)protein captured from
7 CHF corresponds probably to the glycophorin. It is interesting
8 to note that the chromatograms (Figures 5A-C) obtained from the
9 purified glycophorin and the one from CHF plasma were not
10 overlapped. This is due to the fact that the structure of the
11 glycophorin in CHF is maybe slightly modified.

12 To further prove that the captured (glyco)protein is
13 related to glycophorin, the captured (glyco)protein was
14 deglycosylated on chip. Figure 6 shows on-chip deglycosylation
15 treatment of the glycopeptides captured from either purified
16 glycophorin or CHF plasma using the 3F4 monoclonal antibody
17 coated on a PS20 chip. As shown in figure 6, at least 8 major
18 peaks now matched to the peaks generated from the standard
19 glycophorin. Also, it is noted that a lot more peaks were
20 detected, they correspond not only to the peptides but also to
21 the sugar chains released after the deglycosylation treatment.

22 In conclusion, the instant invention provides a sandwich
23 ELISA assay for quantification of a truncated, glycophorin
24 circulating in biological fluid which is diagnostic for CHF. It

1 is important to note that glycoporphin has not been previously
2 recognized as a marker for congestive heart failure (CHF). The
3 instant inventors are the first to document glycoporphin as a
4 marker for CHF and the assay described herein provides an
5 efficient, easy to perform diagnostic method capable of
6 identifying an individual suffering from CHF.
7

8 All patents and publications mentioned in this
9 specification are indicative of the levels of those skilled
10 in the art to which the instant invention pertains. All
11 patents and publications are herein incorporated by reference
12 to the same extent as if each individual patent and
13 publication was specifically and individually indicated to be
14 incorporated by reference.

15 It is to be understood that while a certain form of the
16 invention is illustrated, it is not to be limited to the
17 specific form or arrangement of parts herein described and
18 shown. It will be apparent to those skilled in the art that
19 various changes may be made without departing from the scope
20 of the invention and the invention is not to be considered
21 limited to what is shown and described in the specification.

22 One skilled in the art will readily appreciate that the
23 present invention is well adapted to carry out the objects
24 and obtain the ends and advantages mentioned, as well as
25 those inherent therein. The oligonucleotides, peptides,

1 polypeptides, biologically related compounds, methods,
2 procedures and techniques described herein are presently
3 representative of the preferred embodiments, are intended to
4 be exemplary and are not intended as limitations on the
5 scope. Changes therein and other uses will occur to those
6 skilled in the art which are encompassed within the spirit of
7 the invention and are defined by the scope of the appended
8 claims. Although the invention has been described in
9 connection with specific preferred embodiments, it should be
10 understood that the invention as claimed should not be unduly
11 limited to such specific embodiments. Indeed various
12 modifications of the described modes for carrying out the
13 invention which are obvious to those skilled in the art are
14 intended to be within the scope of the following claims.